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Report

Drosophila Dorsal Paired Medial Neurons Provide a General Mechanism for Memory Consolidation

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Summary

Memories are formed, stabilized in a time-dependent manner, and stored in neural networks. In *Drosophila*, retrieval of punitive and rewarded odor memories depends on output from mushroom body (MB) neurons [1–5], consistent with the idea that both types of memory are represented there. Dorsal Paired Medial (DPM) neurons innervate the mushroom bodies, and DPM neuron output is required for the stability of punished odor memory [6, 7]. Here we show that stable reward-odor memory is also DPM neuron dependent. DPM neuron expression of *amnesiac* (*amn*) in *amn* mutant flies restores wild-type memory. In addition, disrupting DPM neurotransmission between training and testing abolishes reward-odor memory, just as it does with punished memory [7]. We further examined DPM-MB connectivity by overexpressing a DScam variant that reduces DPM neuron projections to the MB α, β, and γ lobes. DPM neurons that primarily project to MB α′ and β′ lobes are capable of stabilizing punitive- and reward-odor memory, implying that both forms of memory have similar circuit requirements. Therefore, our results suggest that the fly employs the local DPM-MB circuit to stabilize punitive- and reward-odor memories and that stable aspects of both forms of memory may reside in mushroom body α′ and β′ lobe neurons.

Results and Discussion

It is widely believed that memory is encoded as changes in synaptic efficacy between neurons in a network. This concept of synaptic plasticity predicts that it will be possible to localize memory to discrete synapses in neural networks in the brain. The relatively small brains of insects are well suited to this endeavor, and genetic manipulation in the fruit fly *Drosophila* has greatly aided neural circuit mapping of odor memory. Flies can be taught to associate an odor conditioned stimulus (CS) with either a punitive electric shock [8, 9] or a rewarding sugar [10] unconditioned stimulus (US). Strikingly, learning and memory with these opposing unconditioned stimuli requires differential transmitter involvement: sugar-rewarded odor memory is dependent on intact octopamine signaling, while shock-punished (punitive) odor memory is dependent on dopamine signaling [4]. However, despite the differential requirement for these monoamine transmitters, blocking MB output during retrieval impairs both punitive- and reward-odor memories [1–5], implying that these memories rely on overlapping brain regions. Here we show that stability of reward-odor memory is reliant on the same MB extrinsic neurons that are required for stability of punitive-odor memory.

amnesiac mutant flies can associate odors with a punitive or a rewarding US, but they quickly forget this information [10, 11], which suggests that *amn* might be generally involved in memory. The *amn* gene is expressed throughout the brain and strongly in Dorsal Paired Medial neurons—two large modulatory neurons that appear to ramify throughout the approximately 5000 neurons of the MBs [6]. We have previously shown that prolonged DPM neuron output is required for the stability of punitive-odor memories [7]. Since DPM neurons heavily ramify in the MBs, these data support the importance of the MB as a crucial locus for memory and also suggested that the neural network involving MB and DPM neurons could be critical for all MB-dependent memory. We therefore tested whether the circuitry involving DPM neurons was involved in the stability of rewarded olfactory memory.

We first confirmed that *amn* mutant flies have a memory defect when conditioned with odors and sugar reward. We used a modified protocol that more closely resembles the odor-shock conditioning protocol and that produces robust memory that lasts for more than 6 hr (Figure 1A). In brief, approximately 100 starved flies were exposed to an odor for 2 min in the absence of sugar, followed by a clean air stream for 30 s and a second odor with sugar reward for 2 min. We tested olfactory memory 3, 60, 180, and 360 min after training. Flies homozygous for the strong *amn* alleles—amn^{1} or amn^{X8}—learn to associate the appropriate odor with sugar reward (they have a small but significant initial performance defect), but they forget this association within 60 min of training (Figure 1A). These data are consistent with the earlier report that amn^{1} flies have defective reward-odor memory [9].

Since *amn* mutant flies forget quickly when trained with either a punitive or a rewarding US, we wondered if similar neural circuitry was involved in both types of memory. Although the *amn* gene is expressed throughout the brain, expressing the *amn* gene in DPM neurons restores punitive odor memory performance to *amn* mutant flies [6, 12]. We therefore tested whether restoring *amn* expression in DPM neurons of *amn* mutant flies would rescue the reward-odor memory defect. We used the c316 {GAL4} line [6] to transgenically express the *amn* gene in DPM neurons of *amn* mutant flies. 3 hr memory of amn^{X8}/amn^{1};c316/uas-*amn* and amn^{1};c316/uas-*amn* flies was similar to wild-type flies and was statistically different from the memory of amn^{X8} and amn^{1};

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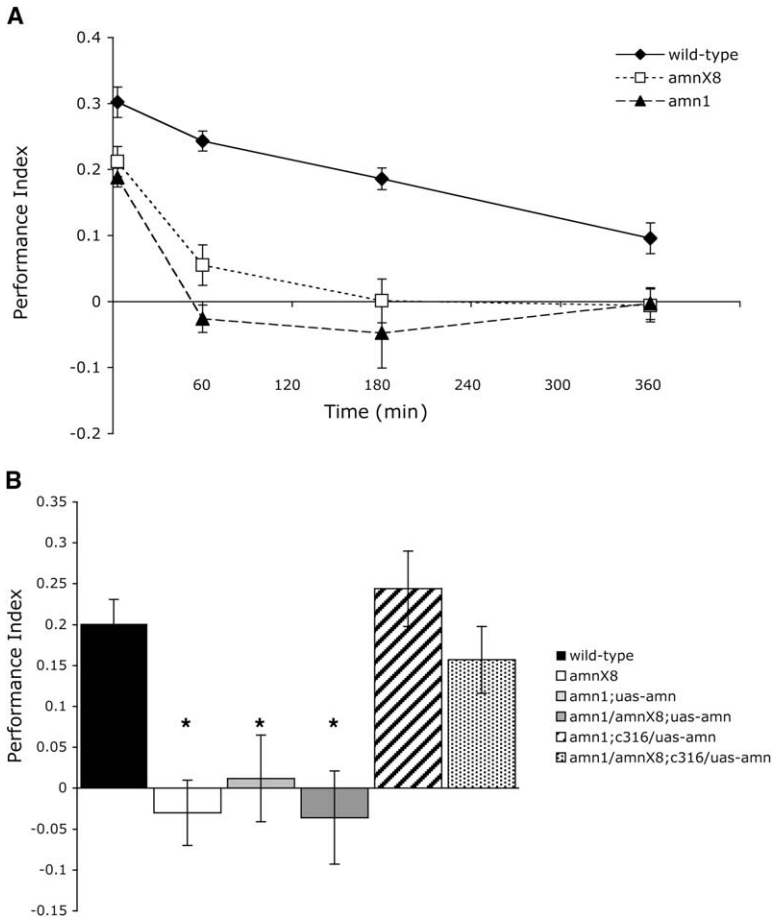


Figure 1. Rewarded Odor Memory Decays Quickly in *amnesiac* Mutant Flies, and Expression of an *amn* Transgene in DPM Neurons Restores Wild-Type Levels of Memory

(A) Wild-type, amn^1, and amn^{X8} mutant flies were conditioned with odor and sugar reward (as described in the main text and Experimental Procedures), and different populations were tested once for odor memory 3, 60, 180, and 360 min after training. *amn* mutant flies have a small but significant initial performance defect ($p < 0.05$).

(B) Wild-type, *amn* mutant, and *amn* mutant flies expressing *amn* in DPM neurons were trained with odor and sugar reward and tested for memory 3 hr after conditioning. Performance of amn^1; c316/uas-*amn* and amn^1/amn^{X8}; c316/uas-*amn* flies was statistically indistinguishable from wild-type flies ($p = 1$ and $p = 0.99$, respectively) and was statistically different from amn^{X8}, amn^1; uas-*amn*, and amn^1/amn^{X8}; uas-*amn* flies (all $p < 0.05$). Error bars are SEM. The asterisks mark groups with statistically different performance from the unmarked groups.

uas-*amn* mutant flies (Figure 1B). These data demonstrate that *amn* expression in DPM neurons is sufficient to restore reward-odor memory to *amn* mutant flies and suggest that DPM neurons are generally critical for olfactory memories.

We next directly tested for an acute role of DPM neurons in reward-odor memory by temporally blocking their output during the course of the experiment (Figure 2). We expressed the dominant temperature-sensitive $shibire^{ts1}$ transgene [13] in DPM neurons by using the c316{GAL4} and Mz717{GAL4} drivers [14] and performed a sugar reward conditioning experiment at either the permissive (25°C) or the restrictive (31°C) temperature. At the restrictive temperature, $shibire^{ts1}$ blocks vesicle recycling and thereby blocks synaptic vesicle release [13]. At 25°C, reward-odor memory of c316; uas-shi^{ts1} and Mz717; uas-shi^{ts1} flies was comparable to memory of wild-type and uas-shi^{ts1} flies. However, at 31°C, memory of c316; uas-shi^{ts1} and Mz717; uas-shi^{ts1} flies was statistically different from wild-type and uas-shi^{ts1} flies. Therefore, DPM synaptic release is necessary for stable reward-odor memory as it is with punitive-odor memory [6, 7].

Stable punitive-odor memory requires prolonged DPM output between acquisition and retrieval, and DPM output is dispensable during training and testing [7]. We therefore tested whether DPM neurons were similarly required for reward-odor memory (Figure 3). We again blocked DPM output by expressing uas-shi^{ts1} with c316{GAL4}, but this time we restricted the inactivation to either the training, testing, or storage period. Blocking DPM neurons during acquisition did not produce memory loss (Figure 3A); memory of c316; uas-shi^{ts1} flies was comparable to wild-type and uas-shi^{ts1} flies. Similarly, DPM neuron output was not required during memory retrieval (Figure 3B); memory of c316; uas-shi^{ts1} flies was comparable to wild-type and uas-shi^{ts1} flies. However, blocking DPM output for 30 min after training significantly reduced reward-odor memory (Figure 3C); memory of c316; uas-shi^{ts1} flies is statistically different from wild-type and uas-shi^{ts1} flies. These data parallel our previous results with punitive-odor memory [7, 15] and suggest that there is a similar requirement for DPM neuron output to stabilize both punitive- and reward-odor memory. We previously reported that DPM block from 30 to 60 min after training decreased punitive-odor memory [15] similar to a 0–30 min block. However, disrupting DPM neuron output from 30 to 60 min had an insignificant effect on reward-odor memory (Figure 3D). These data imply that the role of DPM neurons is diminished at 30–60 min for reward-odor memory. We also used the Mz717 driver to increase the confidence that the temporal uas-shi^{ts1} disruptive effect can be ascribed to blocking DPM neurons (Figure 3E). Blocking DPM output for 60 min after training with Mz717 significantly reduced reward-odor memory. Memory of Mz717; uas-shi^{ts1} flies is statistically different from wild-type and uas-shi^{ts1} flies.

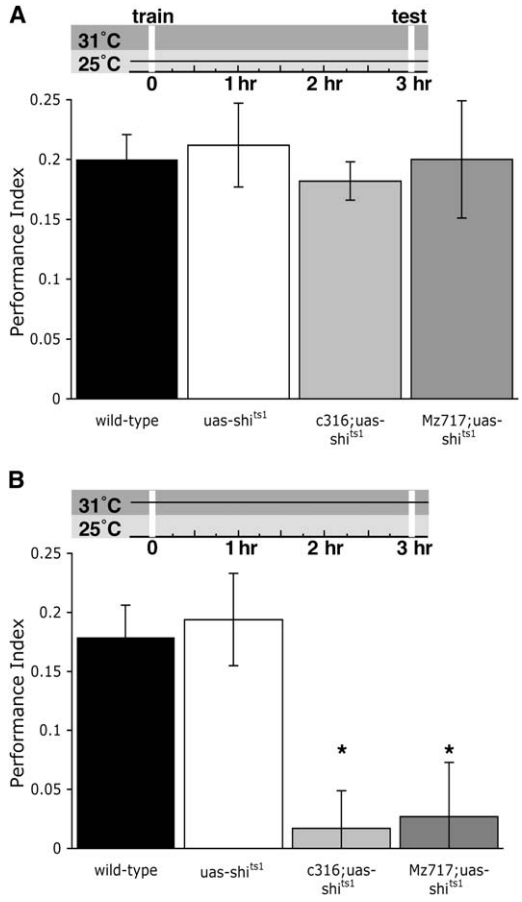


Figure 2. DPM Neuron Output Is Required for 3 hr Rewarded Odor Memory

Temperature shift protocols are shown pictographically above each graph.

(A) The permissive temperature of 25°C does not affect 3 hr reward-odor memory. Performance of c316; uas-*shi*^{ts1} flies ($p > 0.2$) or Mz717; uas-*shi*^{ts1} flies ($p > 0.5$) was indistinguishable from uas-*shi*^{ts1} flies. All genotypes were trained and tested for 3 hr memory at 25°C.

(B) Disrupting DPM output at the restrictive temperature of 31°C abolishes memory. Performances of c316; uas-*shi*^{ts1} flies ($p < 0.0005$) and Mz717; uas-*shi*^{ts1} flies ($p < 0.005$) were statistically different from uas-*shi*^{ts1} flies. All genotypes were trained and tested for 3 hr rewarded odor memory at 31°C. Error bars are SEM. The statistically different groups are marked with an asterisk.

DPM neurons innervate all the lobes of the MBs [6] (Figures 4A–4C), and previous imaging studies suggest that the DPM projections there may be both transmissive and receptive [15]. In addition, expression of n-syb::GFP in DPM neurons has been reported to label DPM projections to the MB lobes [12, 14]. In an attempt to gain further insight into DPM neuron organization, we have overexpressed a collection of pre- and postsynaptic compartment markers in DPM neurons (see Figure S3 in the Supplemental Data available with this article online). However, we see no clear evidence for asymmetry within DPM neurons or between projections to individual MB lobes. Therefore, understanding DPM polarity and organization will require further work.

During this analysis, we found that expression of the DScam17-2::GFP fusion protein (which has been described to label the presynaptic compartment when overexpressed in certain neurons [16]) in DPM neurons, with c316{GAL4}, affected DPM neuron development and resulted in DPM neurons that predominantly project to the MB α' and β' lobe subsets (Figures 4D and 4E, Figures S1D–S1G and S2E–S2H compared with wild-type DPM neurons shown in Figures 4A–4C, Figures S1A–S1C and S2A–S2D). Coexpressing uas-*DScam17-2::GFP* and uas-*CD2* [17] or uas-*lacZ* in DPM neurons reveals that DScam17-2::GFP labels the remaining projections rather than a subset of existing projections (Figures 4D and 4E, Figures S1D–S1G and S2E–S2H). To identify projections to MB α/β neurons versus α'/β' neurons, we costained brains with anti-FASII, which labels α/β and γ neurons [18], and anti-TRIO, which labels α'/β' and γ neurons [19].

A functional role for the MB α' and β' lobes in memory has not been reported. Therefore, we used the uas-*DScam17-2::GFP*; c316 flies to assess the role of DPM neuron projections to the MB α' and β' lobe subset in punitive- and reward-odor memory (Figures 4G and 4H). We included heterozygous uas-*DScam17-2::GFP* flies as a control as well as wild-type and *amn*^{X8} flies for comparison. The presence of the uas-*DScam17-2::GFP* transgene did not significantly affect punitive-odor memory. Remarkably, DPM neurons that primarily project to the α' and β' lobe subsets retain punitive-memory function (Figure 4H). Memory of uas-*DScam17-2::GFP*; c316 flies was similar to memory of uas-*DScam17-2::GFP* flies and was significantly greater than that of *amn*^{X8} flies. Therefore, DPM neuron projections to the α' and β' lobes of the MB are apparently sufficient for punitive-odor memory. We next tested the function of uas-*DScam17-2::GFP*; c316 flies in reward-odor memory. Again, memory of uas-*DScam17-2::GFP*; c316 flies was similar to memory of uas-*DScam17-2::GFP* flies and was significantly greater than that of *amn*^{X8} flies. These data indicate that the DPM neuron projections to the α' and β' MB lobe subsets are also apparently sufficient for reward-odor memory and imply that the circuit requirements for the stability of rewarded and punished odor memory are very similar. Although we cannot currently rule out redundancy of DPM projections or retention of a few critical projections to the α, β, and γ lobes, these data are consistent with the notion that DPM projections to the α' and β' MB lobes are sufficient for stabilizing memory. Our data also suggest that DScam may play a role in wiring the DPM-MB circuit.

In *Drosophila*, there is a striking dissociation of monoamine transmitters for reward and punishment. Dopamine is required for aversive-odor memory formation, whereas octopamine is necessary for appetitive-odor memory [4]. Octopaminergic and dopaminergic neurons project throughout the brain including to the MBs [4, 20, 21]. Although it is not known whether the MB arborization of these monoaminergic neurons is required for odor memories, blocking MB output is required to retrieve both aversive- and appetitive-odor memory [1–5].

DPM neurons ramify throughout the MB lobes and provide a general stabilizing mechanism for both punitive- [6, 7] and reward-odor memory. Our DPM neuron analysis enhances our resolution of memory processing and provides further weight to the idea that components of both punitive- and reward-odor memory reside at synapses within MB neurons [4]. Imaging studies suggest

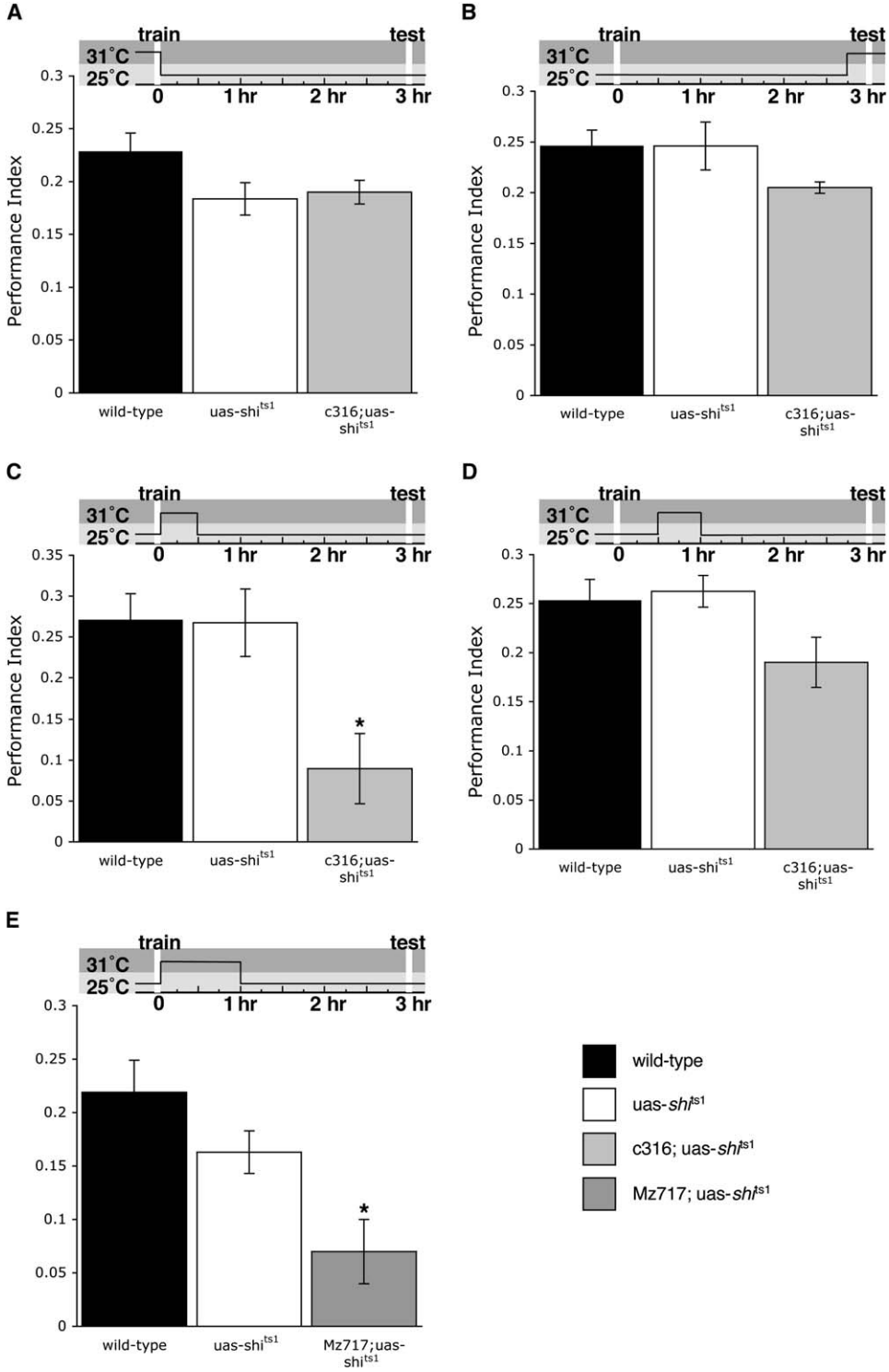


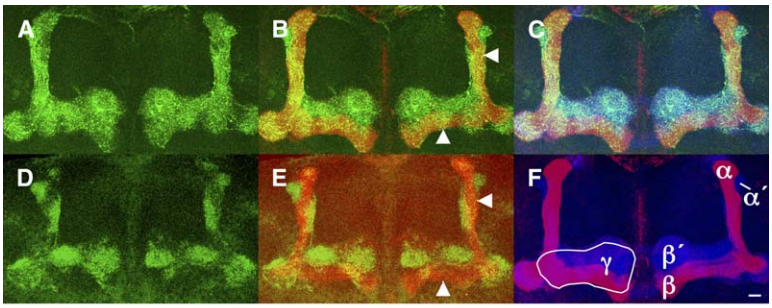
Figure 3. DPM Neuron Output between Training and Testing Is Required for 3 hr Rewarded Odor Memory

The temperature shift protocols are shown pictographically above each graph.

(A) Blocking DPM output during training does not affect 3 hr reward-odor memory. Performance of c316; uas-*shi*^{ts1} flies was indistinguishable from uas-*shi*^{ts1} ($p > 0.9$) and wild-type ($p > 0.1$) flies. Flies were incubated at 31°C for 15 min prior to and during training. Immediately after training, they were returned to 25°C and tested for 3 hr memory.

(B) Blocking DPM output during testing does not affect 3 hr reward-odor memory. Performance of c316; uas-*shi*^{ts1} flies was indistinguishable from uas-*shi*^{ts1} ($p = 0.2$) and wild-type ($p = 0.2$) flies. Flies were trained at 25°C, and 165 min later they were shifted to 31°C. 15 min later, 3 hr memory was tested at 31°C.

(C) Blocking DPM output immediately after training severely impairs 3 hr reward-odor memory. Performance of c316; uas-*shi*^{ts1} flies was statistically different from uas-*shi*^{ts1} ($p < 0.01$) and wild-type ($p < 0.01$) flies. Flies were trained at 25°C, and immediately after training they were shifted to 31°C for 30 min. Flies were then returned to 25°C and tested for 3 hr odor memory at 25°C.



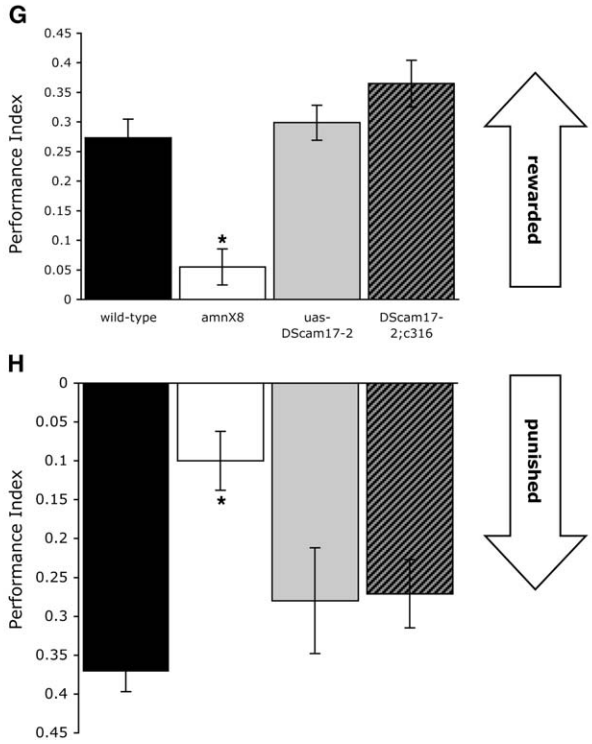


Figure 4. DPM Neurons that Primarily Project to the α′ and β′ MB Lobes Are Functional for Rewarded and Punished Odor Memory

(A–E) Projection views of DPM neuron ramification throughout all the MB lobes. The optical sections with the DPM cell body have been removed to improve visibility of the MB lobe innervation. Single-focal-plane views of the wild-type and DScam17-2::GFP-generated aberrant projections are shown in Figures S1 and S2.

(A) Wild-type DPM neuron projections to all the MB lobes visualized by expressing uas-*CD8::GFP* with c316{GAL4}.

(B) The same wild-type DPM neurons shown in (A) but with the MB α and β lobes (red) counterstained with FASII antibody.

(C) The same wild-type DPM neurons shown in (A) and (B) but with MB α and β lobes (red) counterstained with FASII antibody and α′ and β′ lobes (blue) stained with TRIO antibody.

(D) Expressing a uas-*DScam17-2::GFP* transgene in DPM neurons with c316{GAL4} results in DPM neurons that predominantly project to the MB α′ and β′ lobes. DPM projections are visualized (green) by coexpressing uas-*DScam17-2::GFP* and uas-*CD2* and immunostaining with a CD2 antibody.

(E) The same anti-CD2 labeled uas-*DScam17-2::GFP*-expressing DPM neurons shown in (D) but with the MB α and β lobes (red) counterstained with FASII antibody. Areas where DPM projections to the MB lobes are greatly reduced or absent are marked with arrowheads for comparison with wild-type DPM neurons shown in (B).

(F) Gross anatomy of the MB lobes revealed by FASII (red) and TRIO (blue) immunostaining. In these branched lobes, FASII and TRIO are mutually exclusive. The γ lobe neurons lie along the front of the horizontally projecting lobe subdivision and are labeled by both anti-FASII and anti-TRIO. The MB lobes are symmetrical. Scale bar represents 20 μm.

(G and H) Prime lobe-projecting DPM neurons retain function. Wild-type, *amn*^{X8}, uas-*DScam17-2::GFP*, and uas-*DScam17-2::GFP*; c316 flies were conditioned in either the rewarded (G) or the punished (H) paradigm and were tested for 1 hr memory. Performance of uas-*DScam17-2::GFP* and uas-*DScam17-2::GFP*; c316 flies was statistically indistinguishable from wild-type flies ([G], rewarded memory, uas-*DScam17-2::GFP* $p > 0.95$, uas-*DScam17-2::GFP*; c316 $p > 0.27$; [H], punished memory, uas-*DScam17-2::GFP* $p > 0.7$, uas-*DScam17-2::GFP*; c316 $p = 0.5$) and was statistically different from *amn*^{X8} flies ([G], rewarded memory, uas-*DScam17-2::GFP* $p < 0.0001$, uas-*DScam17-2::GFP*; c316 $p < 0.0001$; [H], punished memory, uas-*DScam17-2::GFP* $p < 0.05$, uas-*DScam17-2::GFP*; c316 $p < 0.003$). Error bars are SEM. The statistically different groups are marked with an asterisk.

that DPM neurons are both receptive and transmissive to MB neurons, and we favor a model where DPM neurons represent recurrent feedback neurons that consolidate conditioned changes in synaptic weight in MB neurons [15] (A.C.K. and S.W., submitted). However, if MB neurons provide drive to DPM neurons, one would expect MB neuron output and DPM neuron output to have similar temporal requirements. Current published data conclude that MB neuron output is not required during memory storage (but is exclusively required for retrieval) [1, 2, 5] when DPM neuron output is required [7]. However, our work here suggests a role for MB α′ and β′ lobe neurons in memory stability, and it is noteworthy that neither MB study [1, 2, 5] employed GAL4 drivers with extensive expression in MB α′/β′ neurons. Further detailed analysis of the role of MB α′/β′ neurons in memory should resolve this conundrum.

Experimental Procedures

Fly stocks were raised on standard cornmeal food at 25°C and 40%–50% relative humidity. The wild-type *Drosophila* strain used in this

(D) Blocking DPM output from 30 to 60 min does not significantly disrupt reward-odor memory. Performance of c316; uas-*shi*^{ts1} flies was statistically indistinguishable from uas-*shi*^{ts1} ($p > 0.05$) and wild-type ($p = 0.1$) flies. Flies were trained at 25°C, and 30 min after training they were shifted to 31°C for 30 min. Flies were then returned to 25°C and tested for 3 hr odor memory at 25°C.

(E) Blocking DPM output immediately after training with the Mz717{GAL4} driver and uas-*shi*^{ts1} severely impairs 3 hr rewarded odor memory. Performance of Mz717; uas-*shi*^{ts1} flies was statistically different from uas-*shi*^{ts1} ($p = 0.02$) and wild-type ($p < 0.0005$) flies. Error bars are SEM. The statistically different groups are marked with an asterisk.

study is Canton-S. The *amn*^{1} and *amn*^{X8} null alleles were described previously [6, 11, 22]. The uas-mCD8::GFP flies are described [23]. The uas-*shi*^{ts1} flies were those previously used by us [6] and first described by Kitamoto [13]. We previously described the DPM neuron-restricted c316{GAL4} and the uas-*amn* flies [6]. The uas-*amn* flies are those previously denoted as "uas-amn#1" [6]. uas-*DScam[exon17-2]::GFP* flies, here designated as uas-*DScam17-2::GFP*, and uas-*CD2* flies were described previously [16, 17].

We generated flies expressing *shi*^{ts1} in DPM neurons by crossing homozygous *w*,uas-*shi*^{ts1};uas-*shi*^{ts1} females to homozygous *w*;c316{GAL4} males. All progeny from this cross carry two uas-*shi*^{ts1} transgenes and one c316{GAL4}. Heterozygous *w*,uas-*shi*^{ts1};uas-*shi*^{ts1} flies were generated by crossing homozygote females to *w* males. A mixed population of sexes was tested in the olfactory conditioning paradigm.

For rescue of the *amn*^{X8} and *amn*^{1} memory defect, we crossed *amn*^{1};c316{GAL4} females with *amn*^{X8};uas-*amn* males. Male progeny from these crosses are hemizygous for *amn*^{1} and heterozygous for c316{GAL4} and uas-*amn*. Female progeny from these crosses are transheterozygote *amn*^{X8}/*amn*^{1} and heterozygous for c316{GAL4} and uas-*amn* . All flies were trained and tested together and sorted after testing and before counting.

We generated flies expressing uas-*DScam17-2::GFP* in DPM neurons by crossing homozygous *w*;c316{GAL4} females with uas-*DScam17-2::GFP*/CyO males. All flies were trained and tested together, and uas-*DScam17-2::GFP*; c316{GAL4} flies were sorted from CyO; c316{GAL4} flies after testing and were counted separately.

The olfactory avoidance paradigm was performed as described previously [7, 9]. The performance index (PI) is calculated as the number of flies avoiding the conditioned odor minus the number of flies avoiding the unconditioned odor divided by the total number of flies in the experiment. A single PI value is the average score from flies of the identical genotype tested with each odor (3-Octanol or 4-Methylcyclohexanol).

We adapted a previously described protocol for olfactory conditioning with sugar reward [4]. Flies were starved for 16–20 hr before conditioning. A conditioning tube (CS+) was made by spreading saturated sucrose (allowed to dry before use) onto a filter paper that covered the entire training tube. Another tube representing the CS− was prepared containing a filter paper soaked in water (and allowed to dry). Approximately 100 starved flies were loaded into the elevator section of a T-maze and trained as follows. Flies were transferred to the CS− tube and exposed to an odor for 2 min. After 30 s of clean air stream, they were transferred back into the elevator and into the sugar reward (CS+) tube, where they were exposed to another odor for 2 min. We tested olfactory memory 3, 60, 180, and 360 min after training. Flies were stored in empty food vials between training and testing. The PI is calculated as the number of flies running toward the conditioned odor minus the number of flies running toward the unconditioned odor divided by the total number of flies in the experiment. A single PI value is the average score from flies of the identical genotype tested with each odor (3-Octanol or 4-Methylcyclohexanol).

We previously determined that the *amn*^{1}, *amn*^{X8}, c316, uas-*amn*, uas-*shi*^{ts1}, and c316; uas-*shi*^{ts1}strains tested in this study have normal odor and electric-shock acuity [6, 7]. We tested odor acuity of both uas-*DScam17-2::GFP* and uas-*DScam17-2::GFP*; c316 flies and confirmed that their acuity is statistically indistinguishable from wild-type flies ($p > 0.5$ for OCT and $p > 0.5$ for MCH) by using previously reported methods [7]. We also determined that the shock reactivity of uas-*DScam17-2::GFP* and uas-*DScam17-2::GFP*; c316 flies was not statistically different to wild-type flies ($p = 0.9$).

We used a variant of the taste-preference assay [24] to measure sugar responsiveness. Flies were starved overnight. Taste preference was assayed on quadrant plates, two of each containing 1% agarose plus or minus 100 mM sucrose. Approximately 60 flies were placed on the plate and allowed to explore the agarose quadrants for 5 min, at which time they were recorded with an iBOT digital camera and BTV Pro software. The number of flies on each quadrant was manually counted at the 5 min time point. A sucrose preference index was calculated as PI = (number flies on sucrose quadrants − number flies on agarose)/(total number of flies). ANOVA revealed no statistical differences ($p > 0.7$) between genotypes used in this study (wild-type, *amn*^{X8}, uas-*shi*^{ts1}, c316; uas-*shi*^{ts1}, Mz717; uas-*shi*^{ts1}, uas-*DScam17-2::GFP*, and uas-*DScam17-2::GFP*; c316). Four groups were analyzed per genotype.

Statistical analyses were performed with KaleidaGraph (Synergy Software). Overall analyses of variance (ANOVA) were followed by planned pair-wise comparisons between the relevant groups with a Tukey HSD post-hoc test. Unless stated otherwise, all experiments are $n \geq 8$.

Adult brains expressing transgenic uas-*mCD8::GFP* or uas-*DScam17-2::GFP* and uas-*CD2* or uas-*lacZ* were removed from the head capsule, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl) for 15 min, and rinsed in PBS-T (PBS containing 0.25% Triton X-100). Brains were incubated with the following antibody concentrations: 1:4 mAb anti-TRIO [19], 1:4 mAb 1D4 anti-FASII [25] (Hybridoma Bank, University of Iowa), 1:3000 Rb anti-FASII (gift from V. Budnik), 1:3000 Rb anti-β-galactosidase (Cappell), 1:200 mAb anti-GFP (Invitrogen), 1:300 Rb anti-GFP(Invitrogen), 1:100 rat mAb anti-HA (Roche), 1:200 mAb anti-Rt CD2 (Serotec). They were then incubated with the appropriate fluorescent secondary antibodies (Jackson Laboratories). Confocal analysis was performed on a Zeiss LSM 5 Pascal confocal microscope. Confocal stacks were processed with ImageJ [26] and Adobe Photoshop.

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/15/1524/DC1/.

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References

1. Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. (2001). Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. Nature *411*, 476–480.
2. McGuire, S.E., Le, P.T., and Davis, R.L. (2001). The role of *Drosophila* mushroom body signaling in olfactory memory. Science *293*, 1330–1333.
3. Schwaerzel, M., Heisenberg, M., and Zars, T. (2002). Extinction antagonizes olfactory memory at the subcellular level. Neuron *35*, 951–960.
4. Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between punitive and appetitive olfactory memories in *Drosophila*. J. Neurosci. *23*, 10495–10502.
5. Davis, R.L. (2005). Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience. Annu. Rev. Neurosci. *28*, 275–302.
6. Waddell, S., Armstrong, J.D., Kitamoto, T., Kaiser, K., and Quinn, W.G. (2000). The *amnesiac* gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. Cell *103*, 805–813.
7. Keene, A.C., Stratmann, M., Keller, A., Perrat, P.N., Vosshall, L.B., and Waddell, S. (2004). Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output. Neuron *44*, 521–533.
8. Quinn, W.G., Harris, W.A., and Benzer, S. (1974). Conditioned behavior in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *71*, 708–712.

9. Tully, T., and Quinn, W.G. (1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. J. Comp. Physiol. [A] *157*, 263–277.
10. Tempel, B.L., Bonini, N., Dawson, D.R., and Quinn, W.G. (1983). Reward learning in normal and mutant *Drosophila*. Proc. Natl. Acad. Sci. USA *80*, 1482–1486.
11. Quinn, W.G., Sziber, P.P., and Booker, R. (1979). The *Drosophila* memory mutant *amnesiac*. Nature *277*, 212–214.
12. Tamura, T., Chiang, A.S., Ito, N., Liu, H.P., Horiuchi, J., Tully, T., and Saitoe, M. (2003). Aging specifically impairs *amnesiac*-dependent memory in *Drosophila*. Neuron *40*, 1003–1011.
13. Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive *shibire* allele in defined neurons. J. Neurobiol. *47*, 81–92.
14. Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D., and Strausfeld, N.J. (1998). The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* Meigen. Learn. Mem. *5*, 52–77.
15. Yu, D., Keene, A.C., Srivatsan, A., Waddell, S., and Davis, R.L. (2005). *Drosophila* DPM neurons form a delayed and branch-specific memory trace after olfactory classical conditioning. Cell *123*, 945–957.
16. Wang, J., Ma, X., Yang, J.S., Zheng, X., Zugates, C.T., Lee, C.H., and Lee, T. (2004). Transmembrane/juxtamembrane domain-dependent Dscam distribution and function during mushroom body neuronal morphogenesis. Neuron *43*, 663–672.
17. Dunin-Borkowski, O.M., and Brown, N.H. (1995). Mammalian CD2 is an effective heterologous marker of the cell surface in *Drosophila*. Dev. Biol. *168*, 689–693.
18. Skoulakis, E.M., and Davis, R.L. (1996). Olfactory learning deficits in mutants for *leonardo*, a *Drosophila* gene encoding a 14–3-3 protein. Neuron *17*, 931–944.
19. Awasaki, T., Saito, M., Sone, M., Suzuki, E., Sakai, R., Ito, K., and Hama, C. (2000). The *Drosophila* trio plays an essential role in patterning of axons by regulating their directional extension. Neuron *26*, 119–131.
20. Riemensperger, T., Voller, T., Stock, P., Buchner, E., and Fiala, A. (2005). Punishment prediction by dopaminergic neurons in *Drosophila*. Curr. Biol. *15*, 1953–1960.
21. Sinakevitch, I., and Strausfeld, N.J. (2006). Comparison of octopamine-like immunoreactivity in the brains of the fruit fly and blow fly. J. Comp. Neurol. *494*, 460–475.
22. DeZazzo, J., Xia, S., Christensen, J., Velinzon, K., and Tully, T. (1999). Developmental expression of an *amn*(+) transgene rescues the mutant memory defect of *amnesiac* adults. J. Neurosci. *19*, 8740–8746.
23. Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451–461.
24. Marella, S., Fischler, W., Kong, P., Asgarian, S., Rueckert, E., and Scott, K. (2006). Imaging taste responses in the fly brain reveals a functional map of taste category and behavior. Neuron *49*, 285–295.
25. Grenningloh, G., Rehm, E.J., and Goodman, C.S. (1991). Genetic analysis of Growth Cone Guidance in *Drosophila*: fasciclin II functions as a neuronal recognition molecule. Cell *67*, 45–57.
26. Abramoff, M.D., Magelhaes, P.J., and Ram, S.J. (2004). Image processing with ImageJ. Biophotonics International *11*, 36–42.